Fungitoxicity and Insecticide Synergism of Monothioquinol Phosphate Esters and Related Compounds. Morifusa Eto*, Yasuaki Hashimoto*1, Kozaburo Ozaki**, Tatsuo Kassai** and Yoshitaka Sasaki** (*Department of Agricultural Chemistry, Kyushu University, Fukuoka and **Division of Phytopathology and Entomology, Kagawa Agricultural Experiment Station, Takamatsu, Japan) Received May 6, 1975. *Botyu-Kagaku*, 40, 110, 1975.

21. モノチオキノールリン酸エステルおよび関連化合物の殺菌性と殺虫剤共力作用 江藤守総*, 橋本恭明*1, 尾崎幸三郎**, 葛西辰雄**, 佐々木善隆** (*九州大学農学部, 福岡市; **香川県農業試験場, 高松市) 50. 5. 6 受理

ハイドロキノン, モノチオキノールおよび関連 (チオ) フェノール類のリン酸エステル14種の殺菌性と殺虫剤共力作用とを調べた. ホスホロチオレートことにモノチオキノールエステルに強い殺菌活性が認められたが, ホスフェートには活性がなかった. これらエステルの加水分解および酸化分解生成物中, チオフェノール, ジスルフィドおよびキノン類には対応するエステルより殺菌力の強いものがあった. ホスホロチオレート類はある程度の殺虫力を有し, またマラソン抵抗性のヨコバイ, ウンカ類に対し, マラソンと共力作用を示し, また抵抗性ハスモンヨトウに対しカルバリルと共力効果を示した. モノチオキノールのS-リン酸O-炭酸エステルが最も強い共力効果を有していた.

In previous papers, we found the metabolic *p*-hydroxylation of triphenyl phosphate, a malathion synergist, and such an interesting biochemical property of quinol phosphates as the inhibition of alcohol dehydrogenase, an SH-enzyme[1,2]. On the other hand, the metabolic pathways of the fungicide edifenphos, S, S-diphenyl ethyl phosphorodithiolate, including *p*-hydroxylationhave been reported[3]. In order to investigate the effect of the *p*-hydroxylation on the biocidal activities of phenyl phosphate and phosphorot hiolate esters, we synthesized a series of phosphorus esters of hydroquinone, monothioquinol and related phenols and examined their biological activities. This paper describes that some phosphorothiolates have fungicidal activity and synergistic activity with malathion against resistant insects.

Materials and Methods

Synthesis

Diethyl 4-ethoxy-2, 3, 5, 6-tetrachlorphenyl phosphate was synthesized by the reaction of chloranil with triethyl phosphite according to Ramirez and Dershowitz[4]. Yield, 35%. M. p., 41-42°C. In the same manner *p*-benzoquinone gave diethyl *p*-ethoxyphenyl phosphate. Yield, 32%. B. p., 150°C (0.1 mmHg). *Anal.* Found: P, 11.31. Calcd. for $C_{12}H_{19}O_5P$: P, 11.26%.

Diethyl *o*-methoxyphenyl phosphate was prepared by the reaction of diethyl phosphorochloridate with *o*-methoxyphenol in the presence of pyridine. Yield, 4%. B. p., 127°C (0.08mmHg). *Anal.* Found: P, 12.36. Calcd. for $C_{11}H_{17}O_5P$: P, 11.90%. Hydroquinone reacted similarly with diphenyl phosphorochloridate to give the diphosphate (13% yield) besides the monophosphate (40% yield) of hydroquinone. The analytical data of the latter was given in our previous paper[1]. The diphosphate, *i.e.* phenylene tetraphenyl bisphosphate, melted at 102-103°C. *Anal.* Found: P, 10.54. Calcd. for $C_{30}H_{24}O_8P$: P, 10.78%.

Diethyl S-*p*-methylphenyl phosphorothiolate was obtained by the reaction of *p*-toluenesulfonyl chloride and three molar equivalents of triethyl phosphite according to Hoffmann's method[5]. Yield, 44%. B. p., 115-130°C (0.15 mmHg). *Anal.* Found: C, 50.75; H, 6.60. Calcd. for $C_{11}H_{17}O_3PS$: C, 50.77; H, 6.54%. Diethyl S-*p*-ethoxycarbonyloxyphenyl phosphorothiolate was synthesized similarly from *p*-ethoxycarbonyloxybenzenesulfonyl chloride and triethyl phosphite. The product was purified through silicic acid column chromatography. Yield, 44%. *Anal.* Found: C, 46.45; H, 5.72. Calcd. for $C_{13}H_{19}O_6PS$: C, 46.70; H, 5.74%.

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For all other phosphorus esters tested except S, S-diphenyl ethyl phosphorodithiolate which was a gift from Nippon Tokushu Noyaku Seizo Co., the methods of preparation and the analytical data were given in the previous paper[2]. All the compounds were purified by recrystallization, distillation, or column chromatography and gave appropriate IR spectra.

Fungitoxicity tests

Aspergillus niger was employed for the test of fungitoxicity. The fungus was cultured for a week at 30°C on the test tube slant medium of pH 5.5 which contained following constituents in gram per liter: KNO_3, 5; KH_2PO_4, 2.5; $MgSO_4$, 1.25; corn steep liquor, 5; sucrose, 25; and agar, 15. The spores harvested were suspended in 50 m*l* of sterile distilled water, followed by dilution four times to get a suspension containing approximately 500,000 spores/m*l*. To the spore suspension was added an equivolume of the medium of same composition described above except for agar.

An aqueous or acetone solution of a test compound was pipetted into a test tube and, after evaporation of the solvent if an acetone solution was used, a suspension of *Asp. niger* spore and sterile water were added to make final volume of 3 m*l*. After 24 hr incubation at 30°C, the inhibition of spore germination was determined[6].

The inhibitory activity of the test chemicals was indicated in the following scale:

− The number of the mycelia were same as blank.
\+ Fewer than blank.
++ Few mycelia elongated.
+++ Complete inhibition of spore germination.

Measurement of oxygen uptake by mycelial suspension

The oxygen uptake was determined by the Warburg technique[7]. *Asp. niger* was grown on the medium described by Watson and Smith[8,9]. A liquid culture was inoculated with a conidial suspension in sterile water obtained from a 4-7-day-slant culture. After incubation at 30°C for 48 hr, the mycelia were harvested, washed four times with a 0.05 M phosphate buffer, pH 7.0, and homogenized in the same buffer to get a mycelial suspension containing 2.7 mg dry weight of mycelia/m*l*. It was incubated at 30°C for 24 hr to get the resting mycelia.

The test system consisted of 1 m*l* of the resting mycelial suspension, 0.1 m*l* of 1.2×10^{-2}M test compound solution in acetone, and 0.3 m*l* of 0.05M phosphate buffer, pH, 7.0. Carbon dioxide evolved was absorbed by 0.2 m*l* of 40% KOH in the center well. After 15 min incubation, 1 m*l* of 0.3M glucose in 0.05M phosphate buffer (pH 7.0) was added from the side arm and oxygen uptake was measured for 30 min at 30°C.

Assay of insecticide synergism

An organophosphate resistant colony NE of green rice leafhoppers, *Nephotettix cincticeps* Uhler, was collected at Nakagawahara, Ehime, in 1963 and has been reared since at the Kagawa Agricultural Experiment Station[10]. Four to five-day-old adult females were topically treated with 0.68 μl of an acetone solution containing malathion and/or a test chemical and kept on rice seedlings under the controlled condition of 16 hr illumination and at 25±1°C. The mortality was counted 24 hr after the treatment[10]. Cotoxicity coefficient was calculated according to Sun and Johnson[11,12].

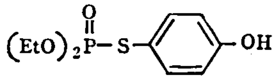
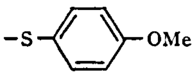
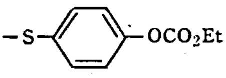
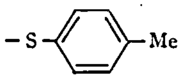
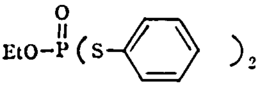
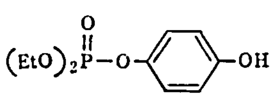
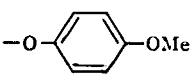
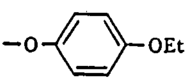
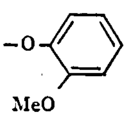
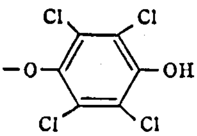
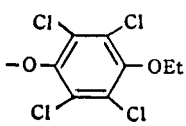
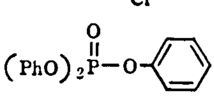
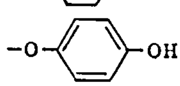
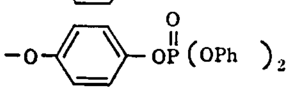
For smaller brown planthoppers, *Laodelphax striatellus* Fallen, the susceptible LE strain and the resistant Rm strain, which was obtained by successive selection with malathion at the Experiment Station, were utilized[13,14]. The dosage mortality data were obtained by a contact method performed with 5 th instar larvae as described previously[14].

A carbaryl resistant colony of tobacco cutworms, *Spodoptera litura* Fabricius, was collected from Takamatsu, Kagawa, in 1970 and has been reared since in the laboratory without insectcide pressure at 25±1°C and 16 hr illumination per day. They were resistant to carbaryl 7-fold. The degree of synergism of carbaryl was assayed by topical application of acetone solutions with and without the test chemicals on 3 rd instar larvae. The treated larvae were kept on synthetic diet under the controlled condition at 25±1°C. Mortality counts were made 24 hr after the treatment.

Measurement of relative partition coefficient

The relative partition coefficient was determined by partition chromatography technique[15,16]. Silica gel thin layer chromatoplate was impregnated with a 5% (v/v) liquid paraffin in hexane

Table 1. Fungitoxicity and inhibitory activity on fungal respiration of phosphorus esters.

Phosphorus esters	Fungitoxicity			Inhibition (%) of respiration at 5×10^{-4}M
	5×10^{-3}M	5×10^{-4}M	5×10^{-5}M	
	+++	+++	++	14
	++	−	−	14
	++	−	−	24
	+++	+	−	15
	++	++	+	28
	−	−	−	2
	+	−	−	24
	+	−	−	
	−	−	−	23
	−	−	−	9
	++	+	−	
	−	−	−	9
	−	−	−	8
	−	−	−	14

and the solvent was evaporated. Acetone-water mixture (3:7 v/v) was employed for development. Phosphate esters were visualized with diazotized sulfanilic acid and phosphorothiolates were with palladium chloride.

Results

Fungitoxic activity

The results of fungitoxicity tests of phosphorus esters are shown in Table 1. Phosphate esters showed little toxicity against *Asp. niger*. Phosphorothiolate esters were generally much more active against the fungus than phosphate esters. Diethyl S-*p*-hydroxyphenyl phosphorothiolate was the most active fungicide among the phosphorus esters tested including the practical fungicide edifenphos, S, S-diphenyl ethyl phosphorodithiolate. The free *p*-hydroxyl group on S-phenyl phosphorothiolates appears to be favorable for the fungitoxicity;the ether and carbonate derivatives were much less active than the parent hydroxy compound. On the contrary, the hydroxyl group in phosphate esters is unfavorable for the activity.

Table 2 shows the fungitoxicity of the hydrolysis and oxidation products of the phosphorus esters. As can be seen in these tables, the fungitoxicity of esters approximately parallels that of their decomposition products, with some exceptions such as sulfonic acids and quinones. Hydrolysis followed by oxidation to disulfides appears to be important for the fungitoxicity of phosphorothiolate esters. Although as described in the previous paper[2] quinol phosphates are readily oxidized to give quinones which have higher fungitoxicity (Table 2), quinol phosphates did not show any appreciable fungitoxicity.

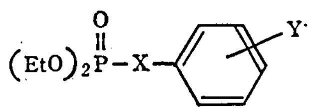
It is presumed from the chemical structure that quinol phosphates may be too polar to penetrate sufficiently into cytoplasm to exert the toxicity. This may be estimated approximately by the partition coefficient. The relative partition coefficients of phosphorus esters are given in Table 3. Phosphorothiolate esters are generally more lipophilic than phosphate esters. Quinol phosphates have very low partition coefficients. This

Table 2. Fungitoxicity and inhibitory activity on fungal respiration of phenols, thiophenols, and related compounds.

Toxicant	Fungitoxicity			Inhibition (%) of respiration at 5×10^{-4}M
	5×10^{-3}M	5×10^{-4}M	5×10^{-5}M	
Thiophenol	+++	++	−	34
p-Hydroxythiophenol	+++	+++	+	33
p-Methoxythiophenol	+++	+++	+	52
p-Thiocresol	+++	+++	+	37
p-Nitrothiophenol	+++	+	−	
Phenyl disulfide	+++	+++	+++	19
p-Hydroxyphenyl disulfide	+++	+++	++	37
p-Methoxyphenyl disulfide	+++	+++	++	30
p-Tolyl disulfide	+++	+++	++	27
Benzenesulfonate*				83
p-Hydroxybenzenesulfonate*	−	−	−	75
p-Ethoxycarbonyloxybenzenesulfonate*	−	−	−	73
p-Toluenesulfonate*	−	−	−	76
Phenol	−	−	−	0
Hydroquinone	−	−	−	0
p-Methoxyphenol	+	−	−	3
p-Benzoquinone	+++	−	−	26
Chloranil	+++	+++	+++	29

* Sodium salt

Table 3. Relative partition coefficient of phosphorus esters.



Ester X	Ester Y	Relative partition coefficient
O	4-OH	0.987
O	2,3,5,6-Cl_4-4-OH	0.752
O	4-OMe	1.874
O	2-OMe	1.481
O	4-OEt	2.788
S	4-OH	9.753
S	4-OMe	2.774
S	4-OCO_2Et	4.495
S	4-Me	3.831
EtO-P(=O)$(SPh)_2$		17.868

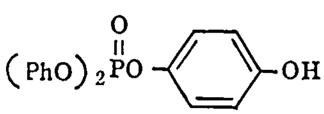
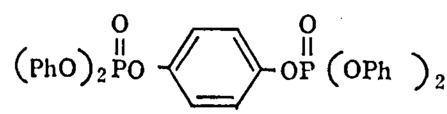
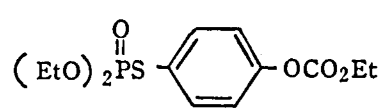
suggests that the esters penetrate poorly inside the cell through lipid layer of fungal cell membrane. On the contrary, S-*p*-hydroxyphenyl phosphorothiolate was more lipophilic than its ether and carbonate ester derivatives..

Table 1 and 2 also show the effect of some phosphorus esters and their hydrolysis or oxidation products on the respiration of *Asp. niger* mycelia. The esters had no appreciable effect but the oxidation products, particularly sulfonic acids, showed considerable inhibitory activity toward fungal respiration. This result is quite different from that of fungitoxicity.

Insecticide synergism

Table 4 shows the results of tests for the synergistic activities of some phosphorus esters with malathion against the resistant (Rm) and susceptible (LE) strains of the smaller brown planthopper, *Laodelphax striatellus* Fallen. The

Table 4. Insecticidal activity (LD_{50} μg/tube) of malathion and the combinations with some phosphorus esters against resistant Rm and susceptible LE strains of smaller brown planthoppers.

Toxicant	Strain Rm Alone[b]	Strain Rm Combination[c] 1:1	Strain Rm Combination[c] 1:5	Strain LE[a] Combination[c] 1:1	Strain LE[a] Combination[c] 1:5
Malathion	41.59				
$(PhO)_3P{=}O$	570.2	11.09 (3.5)[d]	1.79 (17.0)[d]	0.21	0.24
	e	51.76		0.19	
	e	32.73	27.41	0.258	0.376
	124.2	3.59 (8.7)[d]	1.58 (9.8)[d]	0.231	0.094
Piperonyl butoxide		27.17	11.85	0.131	0.142

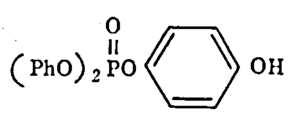
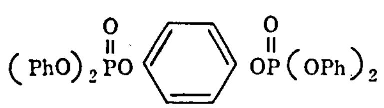
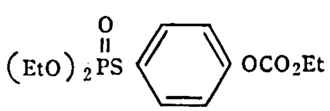
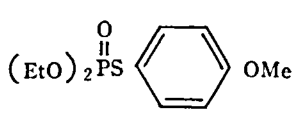
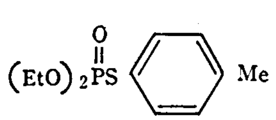
a. LD_{50} of malathion alone was 0.295 μg/tube.
b. LD_{50} toxicant μg/tube.
c. LD_{50} malathion μg/tube.
d. Cotoxicity coefficient.
e. No appreciable toxicity was observed.

strain Rm resisted malathion 141-fold in comparison with the susceptible strain LE. All the tested esters did not show any appreciable synergistic effect against the susceptible strain. Only piperonyl butoxide synergized malathion about twice to this strain. Against the resistant strain Rm triphenyl phosphate showed a 3.5-fold synergistic effect, whereas the quinol analog and the quinol diphosphate had no such activity. On the other hand, the carbonate ester of S-*p*-hydroxyphenyl phosphorothiolate exerted more synergistic activity with malathion at a 1:1 ratio than triphenyl phosphate did. The cotoxicity coefficient of diethyl S-*p*-ethoxycarbonyloxyphenyl phosphorothiolate was 8.7. Piperonyl butoxide increased the toxicity of malathion less than twice against Rm strain at a 1:1 ratio.

When the ratio of synergist: malathion was increased to 5:1, the cotoxicity coefficient of the phosphorothiolate increased a little, whereas that of triphenyl phosphate did extremely as from 3.5 to 17. Piperonyl butoxide synergized 3.5-fold at this ratio.

More phosphorothiolate esters were examined for synergism of malathion against green rice leafhoppers, *Nephotettix cincticeps* Uhler, of the highly resistant Nakagawahara colony and the results are given in Table 5. It resisted malathion about 130 times in comparison with a susceptible colony collected at Kono, Osaka. At a 1:1 ratio of synergist: malathion, triphenyl phosphate increased the toxicity of malathion about twice, whereas the quinol analog and the quinol diphosphate showed only a little synergistic effect. Diethyl S-*p*-ethoxycarbonyloxyphenyl phosphorothiolate, which had a weak insecticidal activity,

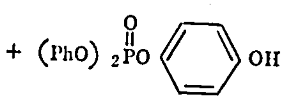
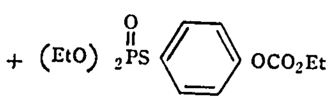
Table 5. Insecticidal activity of some organophosphorus esters and their combinations with malathion against resistant colony of green rice leafhoppers.

Toxicant	LD_{50} toxicant ($\mu g/g$)	LD_{50} malathion ($\mu g/g$) in 1:1 combination	Cotoxicity coefficient
Malathion	606.5		
$(PhO)_3P{=}O$	4600.0	293.7	1.8
	11528.2	462.2	1.4
	9263.1	539.3	1.1
	4216.3	79.8	6.6
	57.1	11.7	4.5
	40.2	8.0	4.7

synergized malathion 6.6-fold. The *p*-methoxy and *p*-methyl derivatives of diethyl S-phenyl phosphorothiolate showed a moderate insecticidal activity by themselves and also synergistic effect with malathion more than 4-fold.

Against a colony of tabacco cutworms, ***Prodenia litura*** Fabricius, which was resistant 7-fold against the carbamate insecticide carbaryl, the synergism of carbaryl was observed with all the tested phosphorus esters including the quinol phosphate. However, the effect of the phosphorus esters were considerably lower than that of piperonyl butoxide as shown in Table 6.

Table 6. Insecticidal activity of 1 : 5 combinations of carbaryl and synergists to resistant colony of tabacco cutworms.

Toxicant	LD_{50} carbaryl $\mu g/g$
Carbaryl	113.89
+ $(PhO)_3P{=}O$	33.26
	45.24
	35.30
+ Piperonyl butoxide	14.72

Discussion

One of the aimes of the series of present research was to elucidate the mechanism of malathion synergism with triphenyl phosphate[1,2,17]. Since triphenyl phosphate itself has only a poor antiesterase activity[1,17], we presumed a hypothetical activation mechanism by biological oxidation: *p*-hydroxylation followed by oxidative transformation into benzoquinone and monomeric metaphosphate. The metaphosphate has been believed to be a very active phosphorylating agent[18]. We found the biological *p*-hydroxylation of triphenyl phosphate[1] and the high inhibitory activity against the SH-enzyme alcohol dehydrogenase of the quinol phosphate and the quinone[2]. However, no synergistic activity with malathion by the quinol phosphate was observed against the malathion resistant strains of green rice leafhoppers and smaller brown planthoppers. Thus, the mechanism of malathion synergism by triphenyl phosphate remained unsolved for future research. The quinol phosphate synergized the carbamate insecticide carbaryl against carbaryl resistant tabacco cutworms, suggesting the inhibition of microsomal oxidative degradation of the insecticide.

In the course of the study on the related compounds, high synergistic activity was found with some S-phenyl phosphorothiolate esters where *p*-substitution of oxygen atom was not necessary to the activity. The S-phenyl phosphorothiolates had also moderate insecticidal activity by themselves. They have high antiesterase activity. For example, I_{50} values for human plasma cholinesterase of diethyl S-*p*-methylphenyl phosphorothiolate and the *p*-methoxy analog were 1.6×10^{-7} and 4.6×10^{-8}M, respectively. Similar insecticidal and anticholinesterase activities of S-aryl dialkyl phosphorothiolates have been reported by Murdock and Hopkins[19]. The high esterase activity has been demonstrated in the malathion-resistant strains of both the insect species[20]. We have found that some organophosphorus esters inhibit certain esterase of resistant leafhoppers and synergize malathion[21,22]. From all of these observations it appears that the synergistic effect of S-phenyl phosphorothiolates may be due to their activity to inhibit certain esterase of the insects.

On the other hand, we found strong fungitoxicity in diethyl S-*p*-hydroxyphenyl phosphorothiolate and some other related phosphorothiolates, whereas such activity was not observed in their phosphate ester analogs. This indicates the importance of P(O)S moiety for fungitoxicity as shown by Kado in the series of benzyl phosphorus esters[23]. In addition to the phosphorothiolate esters, their oxidation products, disulfides, showed strong fungitoxicity, suggesting an activation by oxidative biotransformation. On the other hand, sulfonic acids, i.e. the further oxidation products, showed high inhibitory activity toward fungal respiration, but were almost non-toxic to the fungus. This does not necessarily indicate that sulfonic acids and the inhibition of respiration do not contribute to the fungitoxicity of the phosphorothiolate esters; sulfonic acids may be not able to penetrate into intact fungal cells owing to the high polarity, but to interfer with respiration systems in disrupted cells. Benzenesulfonic acid has been found as a metabolite of S, S-diphenyl ethyl phosphorodithiolate[24].

Although quinones which are the oxidation products of quinol phosphate esters were fungitoxic, the esters did not show the activity. The low partition coefficient of the quinol phosphates may be one of possible reasons why the esters had no appreciable activity. This was supported by the fact that the ether derivatives of quinol phosphates showed some fungitoxicity.

Summary

Fourteen phosphorus esters of hydroquinone, monothioquinol, and related phenols and thiophenols were evaluated for fungitoxicity. Thiolate esters showed generally high activity, but phosphates had no appreciable fungitoxicity. Some hydrolysis or oxidation products of the phosphorus esters were more fungitoxic than the parent esters. They were thiophenols, disulfides, and quinones. Moreover, some S-phenyl phosphorothiolate esters had moderate insecticidal activity and also synergized malathion more effectively than triphenyl phosphate against malathion-resistant strains of green rice leafhoppers and smaller brown planthoppers. The synergism of carbaryl was observed with some phosphorus esters including quinol phosphates against tabacco cutworms.

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